KERNFORSCHUNGSZENTRUM

KARLSRUHE

Mai 1969

KFK 1005

Institut für Strahlenbiologie

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G. Hotz, R. Mauser

GESELLSCHAFT FÜR KERNFORSCHUNG M. B. H.

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Molec. Gen. Genetics 104, 178—194 (1969)

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I. Some Properties of the Spheroplast Assay System

G. Hotz and R. Mauser
Institut für Strahlenbiologie, Kernforschungszentrum Karlsruhe, Germany

Received March 10, 1969

Summary. DNA isolated from coliphage T1 is infective in spheroplasts of *E. coli K12/1*. The efficiency of the assay amounts to approximately 10^{-4} plaque-forming units per DNA molecule of $32 \cdot 10^6$ daltons. A linear relationship between DNA concentration and total phage yield or infective centers, respectively, holds for native DNA. For heat-treated DNA, however, the co-operation of 1.4 molecules is required for successful infection. Beyond a "critical concentration" of about 0.1 μg/ml a self-inhibiting effect of infectious T1-DNA is observed. Breakage by shearing and denaturation of the DNA-molecules destroy their infectious activity. Renaturation, however, restores infectivity to 60—90 per cent of the original activity. Heat treatment of T1-DNA in M/5 NCE buffer results in narrow-coiled, mismatched molecules with partially denatured regions. Though the efficiency of infection of such molecules is reduced by about 30 per cent, the critical concentration of T1-DNA shifts to higher values by a factor of ten, thus giving an increase in the total plaque yield of the system. The effect is explained by the transition of native into narrow-coiled molecular configuration.

Introduction

Biologically active deoxyribonucleic acid has been prepared successfully from a variety of different microorganisms including bacteriophage. Isolated DNA of the latter has been utilized to study problems in molecular biology and especially in radiobiology (Blok *et al.*, 1967; Taylor and Ginoza, 1967; Hotz and Müller, 1968). For elucidation of open questions concerning the action of radiation on DNA, e.g. the contribution of phage protein and of DNA structure to the radiosensitivity or the inactivation of DNA by radiation of small penetrating ability like slow protons or atomic hydrogen, free active DNA molecules are the object of choice. For the understanding of repair mechanisms the use of infectious phage DNA also seems to be promising since the effect of various treatments of the isolated molecules before infection on the repair can be studied.

Concerning efficient infectious systems handling phage DNA several ones are available differing from each other in some respect. The systems of phage ΦX-174 (Guthrie and Sinsheimer, 1963; Sinsheimer *et al.*, 1962) and M13 (Ray *et al.*, 1966) utilize either single- or double stranded DNA of small molecular weight, which is able to infect spheroplasts of *E. coli*. The large DNA molecules of phage lambda, however, need helper phage to infect competent cells (Kaiser and Hogness, 1960) but are also taken up by spheroplasts (Young and Sinsheimer, 1967). DNA of T4 is infecting *E. coli*-spheroplasts in the presence of helper π-particles only (van de Pol *et al.*, 1961). DNA isolated from a phage of *Haemophilus influenzae* (Harm and Rupert, 1963) and DNA of several types of *Subtilis*-phages infect competent cells of their hosts (Romig, 1962; Földes and Trautner, 1964; Green, 1964).

For T-phage, however, which have been used widely in radiobiological experiments, efficient helper-free systems for the assay of infectious DNA are lacking. As to our knowledge only one method has been described in the literature for T1-DNA giving a relatively low efficiency of infection (BRODY *et al.*, 1964). For this and the above-mentioned reason it seemed to be worth-while to improve the assay of the biological activity of isolated T1-DNA. The technique described in this communication is a modification of our method for the spheroplast assay system of ΦX-174 (HOTZ and MÜLLER, 1968) originally developed by GUTHRIE and SINSHEIMER (1963). The efficiency of our assay for infectious T1-DNA appears to be considerably higher than in the assay according to BRODY *et al.* (1964). The first part of our study on infectious T1-DNA gives some data of molecular biological interest. In the second part of this series, which is under investigation, the effect of radiation on the activity of the infectious system will be described.

Material and Methods

Strains. Coliphage T1 wild-type, a host range mutant of this phage, the prototroph *E. coli K12*, and the wild-type of *E. coli B* were originally obtained from Dr. STARLINGER, Köln, in 1958 and 1962, respectively. *E. coli K12/1* is a mutant resistant to T1 and was selected from the wild-type. This strain is used normally as source for the spheroplast preparations. The following strains from which clones resistant to T1 have also been selected, were kindly supplied by Dr. RÖRSCH, Rijswijk: (1) KA16, which is *K12S,hcr*$^-$ from Dr. HARM, Köln. (2) KA63 (= AB2433) which is *HfrH, uvr-C, his*$^-$, *xyl*$^-$, *lac*$^-$, *S*^{r}. (3) KA64 (= AB2434) which is *HfrH, uvr-B, his*$^-$, *xyl*$^-$, *lac*$^-$, *S*^{r}. (4) KA65 (= AB2435) which is *Hfr2, uvr-C, his*$^-$, *xyl*$^-$, *arg*$^-$, *S*^{r}. (5) KA66 (= AB2437) which is *Hfr2, uvr-A, his*$^-$, *xyl*$^-$, *S*^{r}. The strains listed under (2)-(5) originally come from the culture collection of Dr. HOWARD-FLANDERS, Yale University, New Haven.

Preparation of Infectious Phage-DNA. T1-DNA was extracted by phenol from high titer stocks (containing about $5 \cdot 10^{13}$ particles/ml) of concentrated and purified phage. Immediately before extraction of the DNA a CsCl-density gradient centrifugation was performed as the last step of purification. T1 in this highly purified form was found to loose its plaque-forming ability within four weeks by about 50% though it had been extensively dialyzed and kept in an appropriate phage buffer (HERSHEY and CHASE, 1952). The DNA was extracted according to the following procedure: 1 ml of T1 diluted to a final titer of $2 \cdot 10^{12}$/ml in M/15 phosphate buffer is mixed gently with 1 ml of 4% Duponol in a 10 ml centrifuge tube. 2 ml of distilled phenol (75% in demineralized and bidistilled water) is added and the mixture is allowed to stand at 0° C for 30 min. At the beginning and at the end of this period the solution is gently rolled horizontally about the long axis of the glass-stoppered tube for 1 min at a rate of about 25 R.P.M. The phases of the emulsion are separated by centrifugation for 2 min at 3,500 R.P.M. The phenol phase at the bottom of the tube is removed by a syringe and discarded. The extraction is repeated twice. Phenol remaining in the aqueous phase is removed by the addition of equal parts of peroxyde-free ether for 20 min at 0° C. Rolling the mixture again is done as described above. After the time indicated the phases are separated by centrifugation for 0.5 min at 3,000 R.P.M. The ether is removed by a syringe and the procedure is repeated until the aqueous phase is clear which usually takes 4—5 extractions. This step of ether-extraction can be omitted without decreasing the biological activity of the DNA. This procedure even results in a more concentrated DNA-stock. After dialyzing the DNA-solution for 24 hrs against NCE-buffer (0.179 M NaCl, 0.02 M Na-citrate, 10^{-3} M EDTA) 10^{-5} M spermine (Fluka, Switzerland) is added to stabilize the molecules (KAISER *et al.*, 1963). This solution is called the "DNA-stock". Usually it contains about 200 μg DNA/ml as measured by the absorbance at 260 mμ (extinction $\overset{(1\text{ cm})}{(260\text{ m}\mu)} \cdot 47 = \mu$g DNA/ml). Before infection of spheroplasts an at least tenfold dilution is made in Tris buffer (0.05 M, pH 8.1) containing 10^{-5} M spermine. Greatest care is necessary to avoid breakage by shearing. Therefore only pipettes

with cut off tips and an interior diameter of more than 0.9 mm have been used for diluting the DNA solutions.

Preparation of Spheroplasts. Bacteria of *E. coli K12/1* are grown overnight without aeration at 37°C. From this culture 0.04 ml are taken to inoculate 20 ml 3XD-medium (Fraser and Jerrel, 1953). Cells are then grown with aeration to a titer of 10^8/ml (corresponding to an optical density at 550 mμ of 0.25), and centrifuged for 5 min at 4,000 R.P.M. After resuspension of the pellet in 0.35 ml of 1.5 M sucrose the following solutions are added: 0.17 ml of 30% bovine serum albumin (Calbiochem) in 0.25 M Tris (pH 8.1), 0.04 ml of 2 mg/ml lysozyme in 0.25 M Tris (pH 8.1), 0.05 ml of 3.72% EDTA, and 2.5 ml PA-medium (10 g casamino acids (Calbiochem), 10 g nutrient broth (Difco), 10 g glucose, and 100 g sucrose per liter bidistilled water). After the cells have been incubated at 18°C for 15 min 0.05 ml of 10% $MgSO_4$ are added to stop convertion to spheroplasts. Finally 25 μg protamine sulfate (Calbiochem) is added per ml "spheroplast-stock". This spheroplast-stock should be prepared shortly before starting the assay of infectious T1-DNA, otherwise the efficiency of the system is poor.

Assay of T1-DNA. Before taking samples out of the DNA-stock it is always prewarmed at 37°C for 40 min in order to facilitate dissociation of the DNA molecules and hence to achieve a better reproducibility of the system. For infection of spheroplasts 0.4 ml of the stock is added to 0.4 ml of DNA dilution prewarmed as mentioned before. After 20 min incubation at 35°C 0.8 ml of PA-medium containing 0.2% $MgSO_4$ is added and the mixture is held in a water-bath at 35°C for 100 min rocking gently at a frequency of 45/min. From the adsorption tubes a tenfold dilution is made into sterilized, bidistilled water containing chloroform. After 30 min at 18°C mature phage are assayed on coloured agar plates (Bresch, 1952) giving the total phage yield.

For assay of infective centers dilutions and plating have been performed according to the method of Guthrie and Sinsheimer (1963) for phage ΦX-174. The adsorption period was 15 min.

Results

1. Kinetics of the Assay

Fig. 1 (curve A) shows the efficiency of the infection by T1-DNA. It is evident that there exists a linear relationship between the number of phage particles produced by the infected spheroplasts and the number of DNA molecules used in the assay. This linear relationship, however, holds only in the range between 10^6 and $2 \cdot 10^9$ DNA molecules/ml in the adsorption tube. From this linearity of the assay below a concentration of 0.1 μg DNA/ml it is concluded that a single molecule is able to infect a spheroplast of *E. coli K12*. Around concentrations of 10^{10} DNA molecules/ml there is usually a sharp peak of optimal infectivity but sometimes a small plateau can be observed as well. This peak is reproducible and within the limits of experimental error was observed at the same DNA concentration in all preparations made, as can be seen from Table 1.

In all DNA samples tested, however, a rapid decrease in the efficiency of infection is found at concentrations beyond 0.1 μg DNA/ml. This effect could be due to an influence of the medium of the DNA stock solution, to a decrease in the number of phage synthesized per infected spheroplast, or to a decreasing number of spheroplasts infected. The effect was proved to be independent of the buffer used for dialysing the DNA stock. If infected spheroplasts are plated before the end of the latent period the number of infective centers can be measured. However, our observation parallels that made with infectious lambda-DNA (Young and Sinsheimer, 1967). The plaque yield is very low and the results of such experiments are difficult to reproduce. The result of such an experiment is shown in

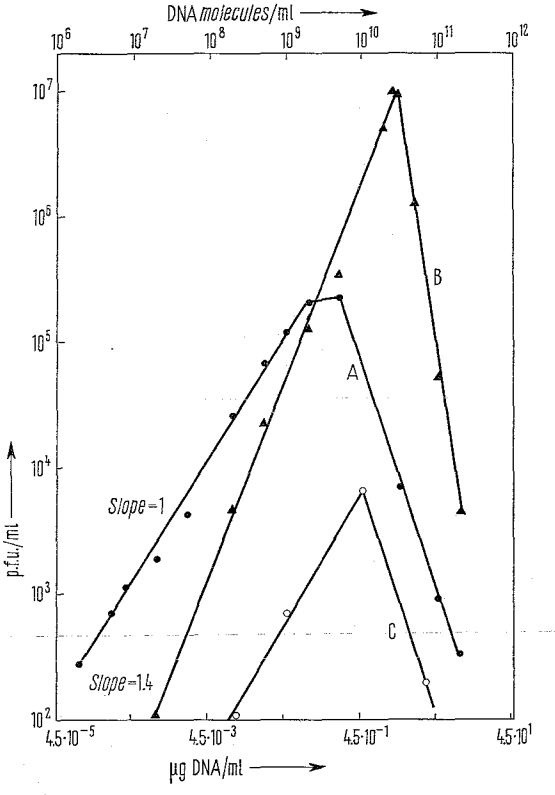


Fig. 1. Number of plaque forming units (*p. f. u.*) per ml in the adsorption tubes as a function of T1-DNA concentration. Infectious DNA was prepared and the spheroplast assay was performed as described in Materials and Methods. Curve A and B show the final plaque yield of mature phage after infection with native (A) and with "heat-treated" (6 min at 100°C in M/5 NCE buffer) DNA (B). Curve C represents the number of infective centers per ml in the adsorption tubes at the end of the adsorption period (15 min)

Table 1

DNA preparation	Concentration of DNA (µg/ml) in the adsorption tube giving maximum yield of phage (curve A in Fig. 1)
7/2/68 I	0.4
7/2/68 II	0.4
10/7/68	0.45
17/9/68	0.45
19/9/68	0.34
15/10/68 I	0.1
15/10/68 II	0.07

curve C of Fig. 1. By comparison of curve A and C it is evident, that: (i) Kinetics of the production of infective centers and mature phage particles are comparable. (ii) At DNA concentrations below 0.1 µg the mean burst size amounts to about 200,

which is in the range of values known from the literature. The burst size decreases, however, by a factor of about ten at high DNA concentrations. From these data we conclude that the rapid decrease in the efficiency of our system at high DNA concentrations, which is expressed by the steep slope of all curves shown in Fig. 1, must be due to a decrease in the number of infected spheroplasts.

Table 2. *Ratio between T1 yield in the presence and in the absence of protamine sulfate during the adsorption period (120 min) with different strains of E. coli K12/1*

KA 16/*1*	$8.2 \cdot 10^2$
KA 63/*1*	$1.2 \cdot 10^3$
KA 64/*1*	$1.6 \cdot 10^2$
KA 65/*1*	$1.2 \cdot 10^2$
KA 66/*1*	$1.8 \cdot 10^2$

Table 2 represents the influence of protamine sulfate (Smull and Ludwig, 1962) on the efficiency of systems with spheroplasts from different bacteria. It is evident from the results that protamine sulfate increases the phage yield remarkably with all strains tested. The efficiency of our system as described above (curve A, Fig. 1) is 10^{-4} mature phage particles produced per one DNA molecule equivalent assuming a weight of $5 \cdot 10^{-17}$ g DNA per T1 phage particle.

2. Influence of Thymus-DNA on the Infectivity of T1-DNA

Several systems do show a competitive effect of unspecific nucleic acids on the efficiency of infectious DNA when present during the adsorption period (Harm and Rupert, 1963; Földes and Trautner, 1964; Young and Sinsheimer, 1967). In all systems a decrease of infectivity was observed beyond a concentration of unspecific DNA or RNA of about 1 μg/ml. However, a decreased efficiency of the assay by concentrated infectious DNA itself, as is observed in our system, was not reported in the literature so far.

Fig. 2 shows the effect of different concentrations of DNA extracted by phenol from thymus tissue on the infectivity of a standard concentration of T1 DNA (0.2 μg/ml). This concentration equals an amount of T1-DNA giving a maximum number of plaques, i.e. corresponding to the peak of the curve A (Fig. 1) of the kinetics. Comparing the results plotted in Fig. 2 with the kinetics of our system shown in Fig. 1 we conclude that at DNA concentrations above 0.5 μg/ml in the adsorption tube, the addition of even small amounts of DNA results in a decreased infectivity. With respect to total DNA concentration the steep portion of the curve in Fig. 2 is comparable to the steep right part of the curve A in Fig. 1. The negative slope of both amounts to 1.6. Obviously, the decrease of infectivity observed at high DNA concentrations is a function of the concentration of macromolecules during the adsorption period and does not depend on the type of DNA, i.e. a solution of homogeneous molecules of T1-DNA at concentrations above 0.1 μg/ml interferes with its own infectivity resulting in a reduced number of infected spheroplasts as proved above. This effect could be due to large DNA molecules hindering each other from penetration through the cell membrane or to a mechanism interfering with phage synthesis when many DNA molecules have entered a spheroplast.

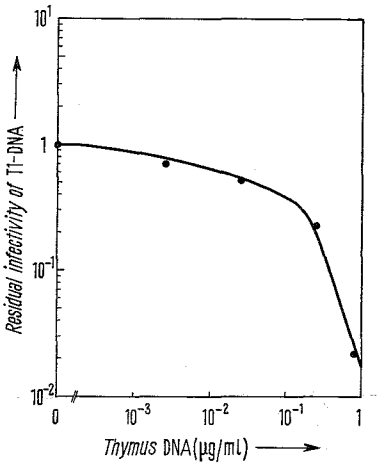


Fig. 2. Effect of heterologous DNA on the efficiency of infectious T1-DNA. Infectivity of native T1-DNA (0.2 µg/ml) is shown as a function of concentration of DNA prepared from calf thymus and added to a standard spheroplast assay

3. Infectivity of T1-DNA after Treatment with Heat and Alkali

Treatment of DNA resulting in strand separation destroys the transforming activity which can be restored to a level as high as about 50 per cent of the original activity by renaturation. This was shown to be the case for transforming DNA of *D. pneumoniae* (MARMUR and LANE, 1960) as well as for other types of transforming and infectious DNA. An exception from the rule is the infectious DNA of several strains of small phages which is even more active, concerning the infection of spheroplasts, as single stranded form than the double stranded RF-DNA.

Table 3 summarizes the results of some experiments with T1-DNA treated for various length of time at 100° C. It is evident from the data listed that:

Table 3. *Effect of heat treatment at 100°C on the infectivity. DNA was heated during the time indicated and thereafter quickly cooled in ice water*

Concentration of DNA during treatment (µg/ml)	Molarity of buffer	Time at 100° C (min)	Relative absorbance at 260 mµ	Concentration of DNA during adsorption (molecules/ml)	Relative infectivity
14	0.01	6	1.28	5×10^9	3.0×10^{-2}
14	0.01 + 2.5% HCHO	6	1.56	5×10^9	$< 10^{-4}$
14	0.01	10	1.33	5×10^9	3.7×10^{-4}
72	0.2	6	1.10	2×10^{10}	77
72	0.2	6	1.10	2×10^9	14
7	0.2	6	1.04	1×10^9	8.5
7	0.2	10	1.16	1×10^9	7.6
7	0.2	15	1.15	1×10^9	6.2

(i) Under our experimental conditions strand separation occurs in a buffer of low molarity what is proved by an increase of the relative absorbance to values usually observed.

(ii) Denaturation inactivates the infectious ability of the DNA to a small fraction. In the presence of formaldehyde during the melting process a high degree of denaturation occurs followed by a complete destruction of infectivity.

(iii) Denaturation in 0.2 M NCE-buffer, however, is poor as was expected, but a very drastic increase in infectivity by a factor of 80 was found. In the range of DNA concentrations tested the increase in plaque counts is proportional to the concentration of infectious DNA in the adsorption tubes. DNA which has received thermal treatment in the way mentioned under (iii), i.e. 6 min at 100° C in 0.2 M NCE, will be called "heat-treated DNA".

Curve B in Fig. 1 represents the kinetics of infection of such heat-treated T1-DNA. DNA in 0.2 M NCES-buffer ($S = 10^{-5}$ M spermine) was heated for 6 min at 100° C in a water-bath, quickly cooled in ice water and thereafter the assay was performed. From comparison of curve B with A and C, respectively, it appears that

(a) The shape of curve B is simular to that of curve A and C exhibiting a sharp maximum separating regions of steep rise and fall.

(b) The beginning of the self-inhibitory effect of T1-DNA at the maximum value, however, is shifted to a higher DNA concentration, i.e. from 0.1 μg/ml in curve A to 1.0 μg/ml in curve B, if the DNA is heat-treated in the way described above. It should be stressed that under these conditions only a small increase of the relative absorbance at 260 mμ is found.

(c) The slope of the left part of curve B amounts to 1.4 and differs significantly from that of curve A and C which is unity. Formally this means that 1.4 molecules of the heat-treated DNA are necessary to give a successful infection of one spheroplast. From this interpretation and from comparison of curve A and B it is quite evident that at low concentrations heat-treated DNA is less infective than an untreated sample. However, the shift of the self-inhibitory effect to higher concentrations results in a drastic increase of the absolute number of plaques in the system at the maximum.

(d) The slope of the right part of curve B, representing the self-inhibitory effect of heat-treated DNA, is increased compared to normal DNA.

Table 4. *Effect of NaOH on the infectivity. (1 part DNA + 1 part 0.2 M NaOH, pH 12.4, 20° C, neutralization with 0.25 M Tris in n HCl. DNA suspended in 0.2 M NCES*

Concentration of DNA during NaOH treatment (μg/ml)	Time at the pH indicated (min)	Relative absorbance at 260 mμ	Concentration of DNA during adsorption (molecules/ml)	Relative infectivity
35	1	1.16	8×10^8	1.6
35	1	1.16	8×10^9	9.0
72	5	1.09	1×10^{10}	33
72	15	1.09	1×10^{10}	42
72	60	1.12	1×10^{10}	29

(e) The relative position of curve B to A seems to be dependent on yet unknown properties of individual DNA preparations, i.e. it is varying from batch to batch.

Table 4 summarizes similar experiments as those listed in Table 3 with the exception that NaOH was used as a denaturing agent. Concerning the relative infectivity left after alkali treatment qualitatively the same result was observed as with heat, i.e. treatment with NaOH under conditions unfavourable to denaturation increases the number of plaques by a factor of about 40. Again, the DNA concentration in the adsorption tube is of limiting influence on this effect.

4. Kinetics of Denaturation and Renaturation of Infectious T1-DNA

In order to obtain more quantitative information on "heat-treated", denatured and renatured infectious T1-DNA we performed a series of experiments which will be described in the following section. First we compared the melting profile under our experimental conditions with the T_m-values from the literature. The absorption of T1-DNA in 0.2 M NCE-buffer at increasing temperature is

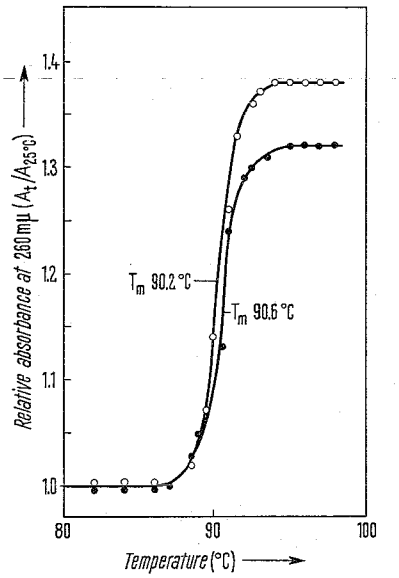


Fig. 3

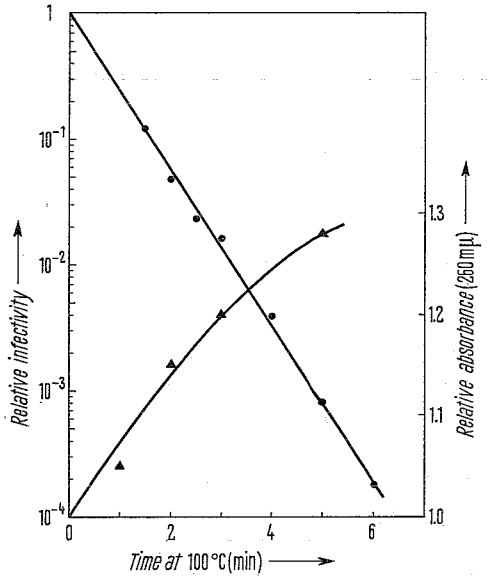


Fig. 4

Fig. 3. Thermal denaturation of T1-DNA as represented by the increase of relative absorbance at 260 mμ. Samples of DNA were diluted to a concentration of 24 μg/ml in M/5 NCE buffer with (●) and without (○) 10^{-4} M spermine added. The absorption was measured at the temperatures indicated

Fig. 4. The effect of heat (100° C) on the infectivity of T1-DNA in M/100 NCES buffer. 0.4 ml of DNA solution (1 μg/ml) were immersed in boiling water for the time indicated plus an additional period of 15 sec for equilibration of the temperature at the beginning. Thereafter the samples were quickly cooled in ice-water and assayed at a concentration of 0.05 μg DNA/ml adsorption fluid. Strand separation during the course of heat treatment is represented by the increase of relative absorbance at 260 mμ

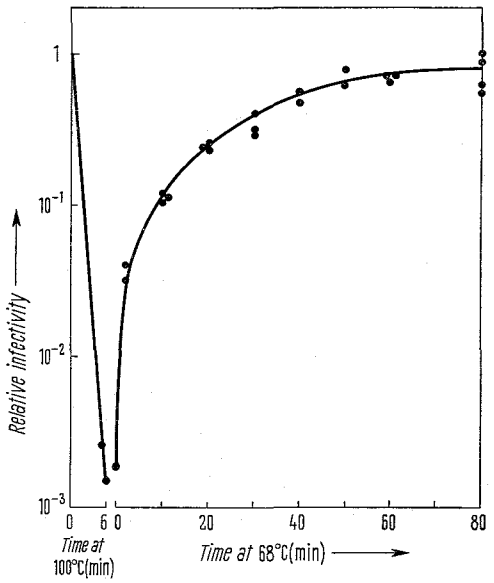


Fig. 5. Restoration of the infectivity of denatured T1-DNA. 10 μg DNA/ml M/100 NCE buffer were heated for 6 min in boiling water and thereafter quickly cooled in ice-water. The relative absorbance at 260 mμ was increased to 1.28 by this treatment. After dilution into M/5 NCE the DNA (5 μg/ml) was immersed in a water-bath at 68° C and portions were withdrawn at the time indicated. The assay on infectivity was performed at a DNA concentration of 0.025 μg/ml adsorption fluid

given in Fig. 3. The characteristics of the curve are unchanged compared to those observed in "SSC" buffer with a molarity of 0.165, usually employed for studies of T_m. There is only a small shift of T_m from 89.5° C (Mora, 1965; Bohne, 1968) in SSC to 90.2° C under our conditions. The presence of 10^{-4} M spermine during denaturation gives an additional ΔT_m of 0.4° C. It is important to note the negligible influence of 10^{-5} M spermine on T_m usually present in our DNA solutions, since in certain systems drastic change of T_m by polyamines has been reported (Mahler *et al.*, 1961; Tabor, 1961).

Fig. 4 shows the effect of denaturation by heat in a buffer of low molarity on the infectivity of T1 DNA. The speed by which inactivation occurs, i.e. the slope of the curve, resembles to results reported for transforming DNA or infectious DNA, e.g. of phage lambda (Alberts, 1968). A fraction of so-called reversibly denaturable DNA, however, could not be observed in our preparations of T1-DNA. Such a fraction would result in a curve with two components.

Fig. 5 gives the results of a typical renaturation experiment using denatured and highly inactivated T1-DNA from a sample like that shown in the preceding figure. It is evident from these data that renaturation at 68° C of the denatured and non-infectious DNA results in a very rapid increase of infectivity. The activity regained amounts to about 60—100 per cent of the original infectivity depending on the DNA concentration during the renaturation procedure. The kinetics of renaturation found with T1-DNA is comparable to those published for DNA from

other sources (MARMUR and LANE, 1960). However, annealing usually restores no more than about 50 per cent of the biological activity before denaturation. The high degree of reactivation of T1-DNA is not simulated by a greater plaque yield due to the heat-treatment effect shown in Fig. 1, curve B, since DNA concentration was kept below $2 \cdot 10^{-2}$ μg/ml during the assay.

5. Effect of Various Treatments on the Infectivity of T1-DNA

The increase in total plaque number per unit weight of DNA described in section 3 is the result of a quite rigorous heat-treatment of concentrated DNA solutions in 0.2 M NCE-buffer. It can be expected that such treatment leads to some change of molecular order, e.g. single strand breaks, double strand breaks, different degrees of "partial renaturation" such as loss of small single stranded fragments, mismatching with loop formation, etc. (SZYBALSKY, 1967). All combinations of such molecular changes may also occur. Furthermore heat-treatment could result in removing protein-DNA crosslinks or in dissociation of molecular aggregates occurring at high DNA concentration. Concerning the effect of such events on the kinetics of our system it should be possible to elucidate some of the configurations mentioned by performing experiments using biochemical and physical methods.

Table 5. *Effect of hydrodynamic shear on infectivity*

Flow rate (ml/min)	Percentage activity remaining
0.75	5.7×10^{-1}
3.3	8.0×10^{-3}
35.0	7.5×10^{-3}

Table 5 lists some qualitative results on the effect of shearing on the infectivity of T1-DNA. Absolute shearing forces have not been measured but rather expressed in relative units. The technique employed was simply to press a constant amount of DNA solution (0.6 ml) through a hypodermic needle (23×0.18 mm). This was done with increasing velocities resulting in increasing shearing forces. Obviously double strand breaks are produced by such a treatment leading to a decrease in infectivity as was expected. We are not able, however, to exclude the possibility that a certain fraction af small pieces of DNA molecules are even more infective than larger ones. This problem could only be solved by measuring the concentration of such hypothetic species.

Table 6 shows the effect of tryptic enzymes on the infectivity of T1-DNA. It is evident from the data that digestion of a small amount of protein left in our DNA preparations after phenol extraction does not result in an increase of the number of total plaques.

If heat treatment of T1-DNA does produce an infective molecule containing partially denatured regions, snake venom phosphodiesterase (SVD) should effect the infectivity of such molecules very dramatically. This enzyme is known to possess exonuclease activity degrading single stranded DNA. From our results

Table 6. *Effect of proteolytic enzymes on the infectivity of T1-DNA. Concentration of DNA during 1 hr of enzymic treatment at 35° C was 30 μg/ml, concentration of enzyme was 5 μg/ml*

Enzyme	Concentration of DNA in the abdsorption tube (μg/ml)	Percentage of activity remaining
Trypsin	0.08	30
Chymotrypsin	0.08	50

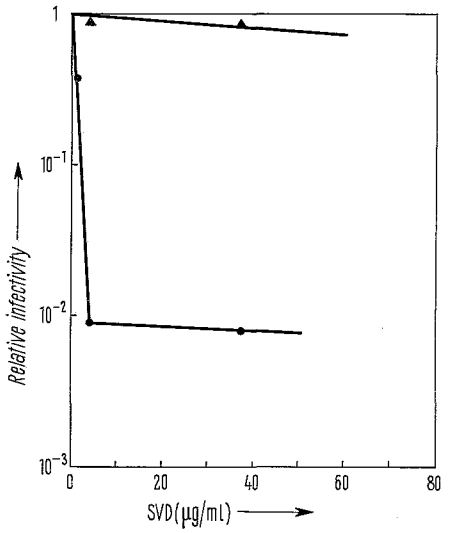


Fig. 6. Effect of various concentrations of venom phosphodiesterase (*SVD*) on the infectivity of native (▲) and heat-treated (●) T1-DNA. Reaction mixture: 0.1 ml DNA (4 μg/ml M/5 NC buffer), 0.2 ml M/10 Tris pH 8.8 containing 10^{-3} M $MgSO_4$ and venom phosphodiesterase (Worthington) of a concentration as indicated. The reaction was allowed to proceed for 4 h at 35° C

shown in Fig. 6 we conclude that native DNA is not attacked by SVD. DNA molecules still infective after heat treatment, however, are degraded by the enzyme. Consequently, their molecular configuration is consistent with a structure containing single-stranded regions including free ends.

6. Comparative Morphology of Native, Heat-Treated, and Denatured T1-DNA

The electron microscope was used for further elucidating the structure of infectious T1-DNA before and after thermal treatment. Fig. 7a gives a typical example of our native DNA preparation which forms, as commonly observed, long random-coiled filaments. Most of the molecules have a length between 15 and 17 μ corresponding to a molecular weight of $30—34 \cdot 10^6$ daltons. Both values are well in agreement with data recently measured by analytical ultracentrifugation (Bresler *et al.*, 1967; Bohne, 1968) and electron microscopy (Lang *et al.*, 1967).

Denatured T1-DNA appears as more compact coils and is shown in Fig. 8a as has been reported previously. The characteristic structure of T1-DNA mole-

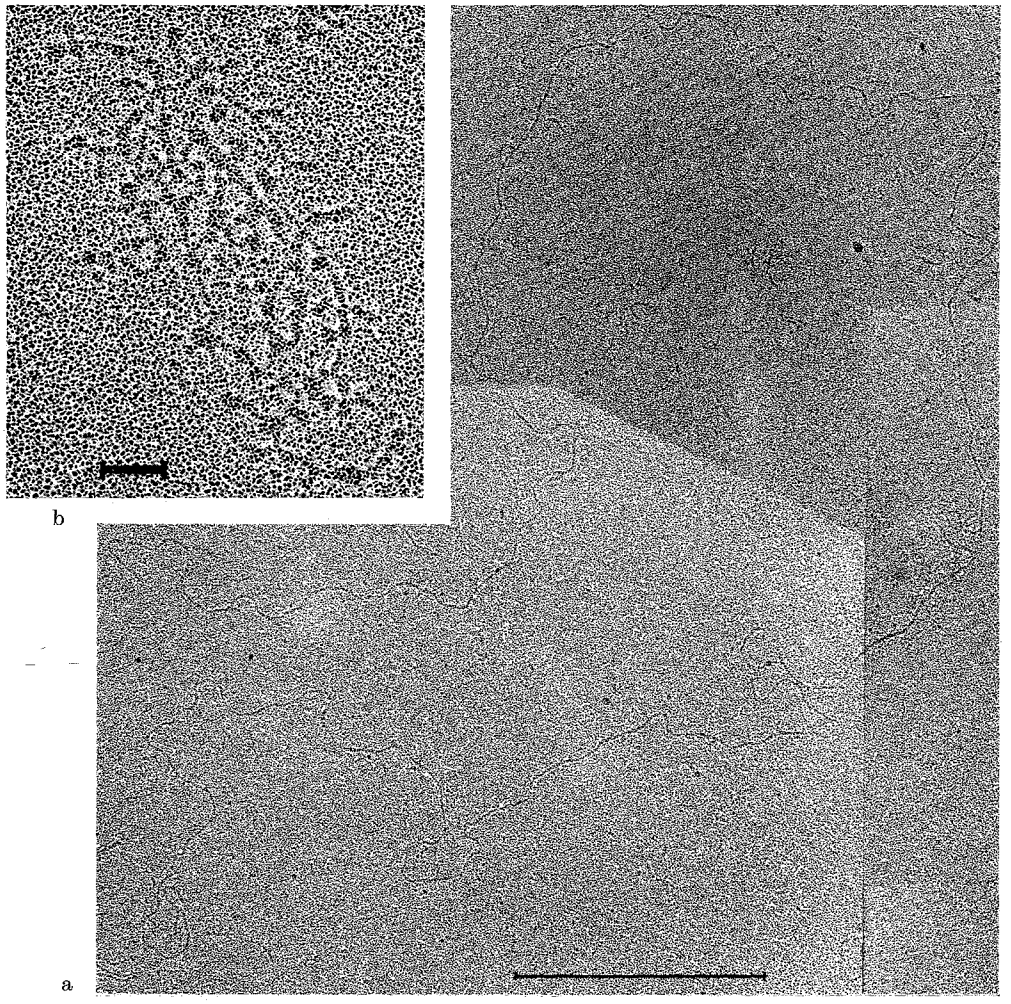
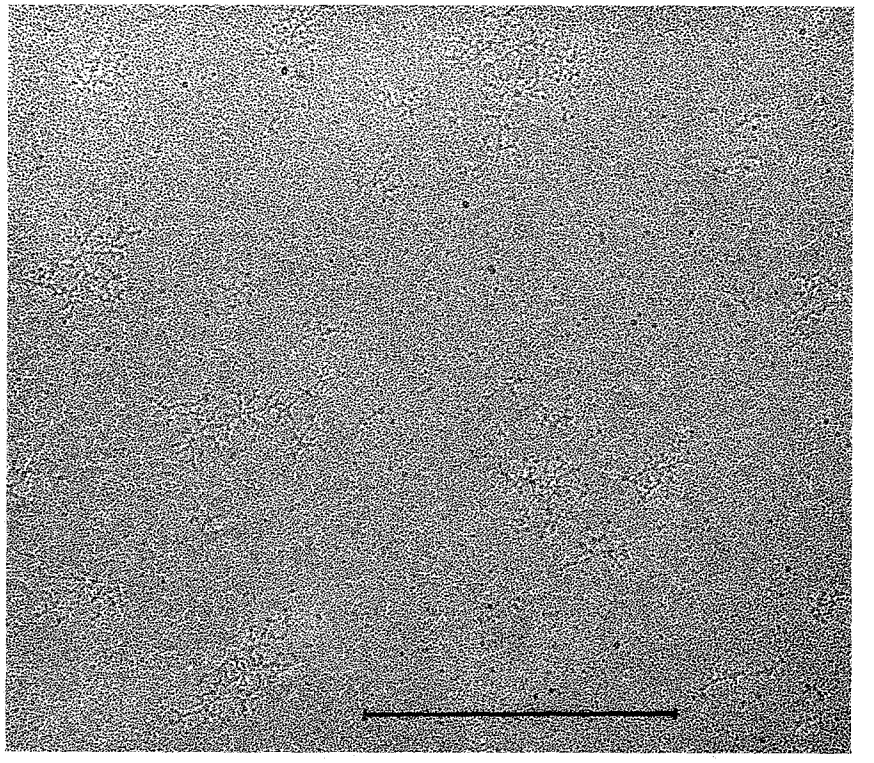
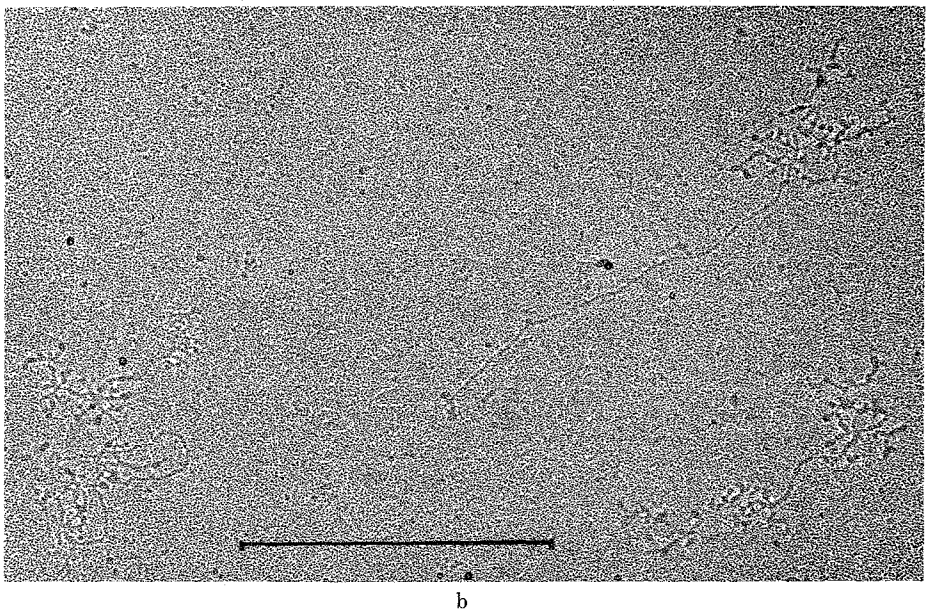


Fig. 7. a Native T1-DNA. This and the following samples were prepared according to the Kleinschmidt-technique at a DNA concentration of 2 μg/ml and photographed by Dr. H.FRANK (Max-Planck-Institut für Virusforschung, Tübingen). In this and all succeeding illustrations the scale mark represents 1 μ, if not stated otherwise. b "Heat-treated" T1-DNA molecule, enlarged detail of Fig. 9. Scale mark represents 0.1 μ

cules from a preparation, which is still infective after a heat treatment of 6 min at 100°C in 0.2 M NCES, however, is quite different from the two configurations mentioned before. From the Figs. 7b, 8b and 9 it is obvious that the DNA molecules are partially collapsed, but still contain many double stranded regions of various lengths. Sometimes long filaments of stiff native-like double strands have ends which are denatured or at lesat mismatched with loop-like formation. Whole native-like molecules are not seen in such preparations. The microscopic pictures are completely consistent with a DNA structure, partially single-stranded which can be degraded by phosphodiesterase. From our experiments with SVD described



a



b

Fig. 8. a T1-DNA denatured by heat (non-infective). b "Heat-treated" T1-DNA (infective)

Fig. 9. "Heat-treated" T1-DNA (infective)

in the foregoing section we did expect a partially denatured or partially renatured DNA molecule which, however, should still be infective for *K12*-spheroplasts. Transforming molecules of *Haemophilus influenzae* DNA with similar secundary structure appearing as a small fraction after denaturation have recently been described (Chevallier and Bernardi, 1968).

Discussion

It has been shown that spheroplasts of *E. coli K12/1* can be infected successfully by DNA extracted from phage T1. The essential qualities of this system derived from our experiments can be summarized and discussed as follows:

(i) The system described may be called a "simple two-component assay" since it consists only of phage DNA and spheroplasts while there is no need of adding helper phage particles. The efficiency of the assay amounts to approximately 10^{-4} plaque forming units per DNA molecule which is in the range obtained with other comparable systems, e.g. that of phage lambda (Young and Sinsheimer, 1967).

(ii) A linear relationship between concentration of infectious DNA and total phage yield or infective centers, respectively, is observed. This relationship, however, holds only up to a concentration of about 0.1 μg DNA/ml in the adsorption tube. Beyond this "critical concentration" the total phage yield of the system as well as the number of infective centers decrease rapidly. This result is consistent with the idea that T1-DNA molecules at high concentration are self-inhibiting their ability to infect spheroplasts. This hypothesis is confirmed by the observation of a blocking effect of calf thymus DNA upon the assay.

(iii) Breakage of native molecules by shearing destroys their infectivity. Assuming that degraded DNA is taken up by spheroplasts with unchanged efficiency it may be concluded that this DNA is not able to carry complete genetic information.

(iv) Single stranded T1-DNA obtained by denaturation is not able to initiate phage production in the system. It is not yet understood whether single strands are unable to penetrate the membrane of a spheroplast or whether their genetic information for synthesis of mature phage is incomplete.

(v) Infectivity of T1-DNA is lost after denaturation but it can be restored by annealing to an unexpected degree, i.e. between 60 and nearly 100 per cent of the original activity. This phenomenon seems to be related to the unusual resistance of the genetic information of T1-DNA against heat treatment discussed under (vi). It should be stressed, however, that the efficient restoration of infectivity after annealing is not due to an increase of total plaque yield after heat treatment discussed below.

(vi) The "critical concentration" of T1-DNA in the kinetics can be shifted towards higher concentration by a factor of about ten after heat treatment of concentrated DNA for 6 min at 100°C in M/5 NEC buffer and subsequent rapid cooling. Under such conditions "renaturation" of DNA, i.e. restoration of infectivity occurs to a level beyond 100 per cent. Though the total phage yield of the system increases after this treatment in some preparations by a factor of about 80 this is not caused by an increase of efficiency of the system. From the

slope of the total phage yield versus DNA concentration it is evident that 1.4 molecules of "heat-treated" DNA are necessary for infection instead of only one molecule of native DNA. From these findings we conclude that the enhancement of total plaque yield produced by the heat treatment applied cannot be due to the formation of a new kind of DNA molecules which are more infective than native ones. As has been shown above the application of electron microscopy revealed the formation of irregular DNA molecules under these conditions. We assume that transition of the native molecule with a large end-to-end distance into a narrow-coiled molecule is responsible for the shift of the critical DNA concentration in the kinetics of heat-treated DNA, since such coiled DNA molecules should have a reduced probability in hindering each other during the process of infection at high DNA concentration.

Considering an efficiency of infection between 10^{-4} and 10^{-6} it is obvious that electron microscopy cannot supply representative pictures of the small fraction of infective molecules. On the other hand it seems to be justified to expect infective molecules to behave identical to the mass of DNA concerning denaturation, renaturation, and mismatching. This is supported by our findings that heat-treated infective molecules contain single strand regions which can be digested by phosphodiesterase. The efficiency of infection is reduced by heat treatment by only about 30 per cent compared with native ones. It is most surprising that mismatched molecules containing single stranded sections with free ends are still infective. We attribute the small increase of absorbancy at 260 mμ observed in the heat-treated samples to these partially denatured regions.

Summing up it seems to us that the most striking feature of infectious T1-DNA is the ability to preserve its complete information and its viability even after most rigorous heat treatment resulting in transition of the native molecular configuration to a narrow-coiled double stranded DNA including partially denatured regions. Further studies are planned to show whether infectious T1-DNA can be used as a suitable system for elucidating questions of the transfer of genetic phage information to the; host by various modifications of phage-DNA.

Acknowledgements. The authors gratefully acknowledge the contribution of Dr. H. FRANK (Max-Planck-Institut für Virusforschung, Tübingen, Germany), who performed the electron microscopy of our DNA preparations. Drs. G. BERNARDI and T. A. TRAUTNER we thank for fruitful discussions.

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Communicated by P. Starlinger

Dr. Gerhart Hotz
Institut für Strahlenbiologie
Kernforschungszentrum Karlsruhe
D 75 Karlsruhe 1, Postfach 3640